IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Jeffery P. Erickson		
Serial No.:	10/505,191	Art Unit:	1632
Filed:	06/24/2005	Examiner:	Sgagias, M.
Entitled:	**Anticancer Compounds And Methods**		

DECLARATION OF DR. THOMAS WHEELER UNDER 37 CFR § 1.132

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I hereby certify that this correspondence (along with any referred to as being attached or enclosed) is, on the date shown below, being deposited with the U.S. Patent and Trademark Office, via EFS.

Dated: February 11, 2008 By: Tracy E. Light

Examiner Sgagias:

I, Thomas Wheeler, Ph.D. under penalty of perjury, state that:

1. I am an employee of AgResearch, Ruakura Research Centre, Hamilton, New Zealand. I am considered an expert in the field of salivary proteins, particularly those of farm animals.

2. I first identified and sequenced the bovine salivary protein (bSP) gene and protein in the mid-1990's. In particular, I sequenced two versions of the bSP30 gene (Version A and Version B). These sequences were made available to the public

in 1996 and published as Accession Number U79413 (Version A) and Accession Number U79414 (Version B). See, Tab A & Tab B, respectively.

3. At the same time, I co-authored a publication describing the electrophoretic isolation of the bSP30 protein and generated a preliminary amino acid sequencing analysis of bSP30. Rajan et al., "The relative abundance of a salivary protein, bSP30, is correlated with susceptibility to bloat in cattle herds selected for high or low bloat susceptibility" *Animal Genetics* 27;407-414 (1996). See, Tab C. In this publication, we suggested that genetic differences in the bSP30 promoter may be responsible for increased bSP30 expression in high bloat susceptible cattle. Further, I concluded that bSP30 salivary proteins were: i) relatively abundant in the parotid gland; and ii) not related to the acidic proline-rich proteins. This suggested that bSP30 was a parotid salivary protein (PSP) as it is now categorized.

3. Shortly thereafter, a genealogical analysis of the bSP30 nucleic acid and amino acid sequences confirmed their relation to other parotid salivary proteins (PSPs). Wheeler et al., "The BSP30 salivary proteins from cattle, LUNX/PLUNC and von Ebner's minor salivary gland protein are members of the PSP/LBP superfamily of proteins" Biochim Biophys ACTA 1579:92-100 (2002). See, Tab D. In this publication, I affirmed the hypothesis inherent in the Rajan et al. 1996 publication that the bSP30 proteins were structurally and functionally similar to PSP proteins such that they can be considered part of the same family.

4. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing therefrom.

Dated: February 7, 2008

Dr. Thomas Wheeler

☐ **1:** U79413. Reports ...[gi:1710366] The record has been replaced by 108744293

Comment Features Sequence

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            of BSP30, a bovine member of the Parotid Secretory Protein family
  JOURNAL   Unpublished
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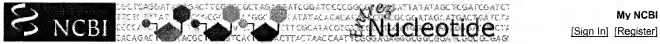

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Features Sequence

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DEFINITION  Bos taurus common salivary protein BSP30 mRNA, form b, complete
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ACCESSION   U79414
VERSION     U79414.1  GI:1710368
KEYWORDS    .
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REFERENCE   1  (bases 1 to 1024)
  AUTHORS   Haigh,B.J., Wilkins,R.J. and Wheeler,T.T.
  TITLE     The cloning and sequencing of two cDNAs coding for alternate forms
            of BSP30, a bovine member of the Parotid Secretory Protein family
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1024)
  AUTHORS   Haigh,B.J., Wilkins,R.J. and Wheeler,T.T.
  TITLE     Direct Submission
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. nimal Genetics, 96, 27, 407–414

The relative bundance of a salivary protein, bSP30, is correlated with susceptibility to bloat in cattle herds selected for high or low bloat susceptibility

G H Rajan, C A Morris, V R Carruthers, R J Wilkins, T T Wheeler

Summary

Pasture bloat is a serious economic and animal welfare problem in cattle grazed on legumes in New Zealand. Analysis of salivary proteins from dairy cattle in herds bred for either low or high susceptibility to bloat has resulted in the identification of a 30 kilodalton protein, which we term bSP30, whose relative abundance is negatively correlated with bloat score ($r = -0.40 \pm 0.12$). From 74 animals sampled, relative abundance of bSP30 was 66 ± 15% higher in the low-susceptibility herd than in the high-susceptibility herd. Relative abundance of bSP30 also varied significantly within individuals, according to feeding or time of day, and from day to day. A sequence homology search of 38 amino acids derived from three tryptic fragments of the protein suggests that the amino acid sequence of bSP30 has not been described previously. Amino acid analysis indicates that bSP30 is not a member of the proline-rich family of salivary proteins. The function of bSP30 is unknown but it is conceivable that it plays a role in the aetiology of bloat.

Key words: bloat, bovine, electrophoresis, salivary proteins, susceptibility

Introduction

Foamy bloat is a disorder of cattle in which the rumen contents form a stable foam under certain conditions, typically when the animal has grazed on clover. This results in an inability to expel gas produced by bacterial fermentation in the rumen. Gas pressure expands the rumen to such an extent that in severe cases the animal cannot breathe and so asphyxiates. The disorder has considerable economic impact (NZ$ 50 million per annum), requiring twice-daily prophylactic drenching of cattle with antifoaming agents, for at least 1–3 months of the year, on many New Zealand dairy farms (Carruthers *et al.* 1987).

Considerable variation is observed among animals in their propensity to bloat and this variation seems to be a result of physiological rather than behavioural differences (Cockrem 1975; Cockrem & McIntosh 1976). However, the causative physiological difference between bloating and non-bloating animals, grazing on the same pasture, is not known. Susceptibility to bloat, as measured on a subjective scale (bloat score), appears to be an inherited characteristic. Two cattle herds were established at Ruakura in 1972 from animals having low or high bloat scores. Successive generations of within-herd selection have resulted in a marked divergence of mean bloat susceptibility between the low susceptibility (LS) and high susceptibility (HS) herds (Morris *et al.* 1991). These herds provide a resource in which to determine the physiological basis for differences in bloat susceptibility as well as to investigate the genetics of bloat susceptibility.

The identification of a physiological or genetic marker for bloat susceptibility would enable the screening of dairy herds and sires, with a view to decreasing susceptibility in the commercial population by selective breeding. Saliva composition or production rate could conceivably be different between LS and HS animals because saliva is a major contributor to the rumen contents. Indeed, saliva flow rates have been shown to be significantly greater in LS than in HS cattle (McIntosh & Cockrem 1977). Bovine salivary proteins have been analysed by electrophoresis. However, for the most part this has been restricted to native proteins (McLaren *et al.* 1987). The first objective of the present study was to identify and evaluate salivary protein variation between the Ruakura LS and HS bloat herds using SDS electrophoresis. Having found a protein whose relative abundance was 66 ± 15% higher in saliva from cattle from the LS herd, some non-genetic factors affecting its abundance were studied. The correlation of the abundance of this protein in saliva with susceptibility to bloat was estimated. Finally, this protein, which

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we call bovine salivary protein 30 kDa (bSP30), was partly characterized by two-dimensional electrophoresis, amino acid sequencing and amino acid composition analysis.

Materials and methods

Saliva collection, sample preparation and electrophoresis

Parotid saliva was sampled by placing a hollow bit in the mouth above the tongue near the opening of the parotid salivary duct (McIntosh *et al.* 1988). Saliva was collected over 3 min by applying suction with a vacuum pump. The bit was washed between sample collections. After collection, saliva was centrifuged at 1500 *g* for 10 min to sediment particulates and stored in aliquots at −70°C. The protein concentration of the saliva was assayed by the method of Bradford (Bradford 1976), using commercially supplied reagent (Bio-Rad, Hercules, CA). Saliva was lyophilized and reconstituted in a solution containing 0·0625 M Tris.HCl (pH 6·9), 5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, 10% (w/v) glycerol and 0·01% (w/v) bromophenol blue, to a final protein concentration of 1 mg/ml. Aliquots of 30 µl were loaded into wells of a 1·5 mm x 16 cm x 20 cm SDS polyacrylamide gel, prepared and run according to the method of Laemmli (1970). The separating gel contained 12% (w/v) polyacrylamide and the ratio of acrylamide monomer to bis cross-linker was 30:1 by weight. Gels were subjected to electrophoresis at 30 mA/gel until the bromophenol blue was 1–2 cm from the bottom of the gel. The gels were then soaked overnight in a solution containing 0·25% (w/v) Coomassie brilliant blue G-250 (Bio-Rad), 50% (v/v) methanol and 10% (v/v) acetic acid, and destained in several changes of a solution containing 40% (v/v) methanol and 5% (v/v) acetic acid.

Two-dimensional electrophoresis on large-format gels was performed as described previously(Voris & Young 1980; Young *et al.* 1983).

Quantification of salivary proteins after electrophoresis

After destaining, proteins present in the gels were quantified by laser scanning densitometry using a scanner and image analysis software (ImageQuant; Molecular Dynamics, Sunnyvale, CA). The proteins were quantified using a 'broad line' approach as suggested by the manufacturer. In this approach, a rectangle is defined, which is narrower than the width of the lane and runs the length of the lane. The density is averaged horizontally across the width of the rectangle for each data point down the length of the rectangle, thus providing a density profile down the length of the lane. The integrated density of each protein band is calculated by determining the area under the curve in the density profile. The relative abundance of bSP30 was then obtained by dividing the integrated density of the bSP30 band by the sum of the integrated densities of all the protein bands detected in the same lane.

Determination of susceptibility to bloat and bloat score

All animals were scored for bloat at 6 months of age. In total, 74 animals (46 HS and 28 LS) were tested. Animals were grazed as a herd on lush pastures containing predominantly white and/or red clover for a period of 3 weeks. The LS and HS animals were grazed together on the same field. During this time the animals were scored for bloat twice daily on a 0–4 scale of severity (Johns 1954) where 0 = no bloat, 1 = mild bloat, 2 = moderate bloat, 3 = severe bloat, 4 = treatment required to prevent death. From 1 upwards, half-units were used. The bloat scores for each of the animals was averaged over all the 'qualifying half-days'. A qualifying half-day was defined as one in which at least 20% of the HS animals scored 2 or greater. The average bloat score per animal was adjusted for fixed effects as described below.

All procedures involving animals met the ethical standards set by the Ruakura Animal Ethics Committee.

Feeding regimens applied

The variability of bSP30 abundance, both within and between days, was tested in four LS and four HS cows subjected to the following routine. The animals were grazed on limited forage overnight so that they fed simultaneously on fresh pasture when it became available. The cows were fed *ad libitum* on ryegrass/white clover pasture from 7 am to 9 am, after which they were removed from pasture and the first saliva sample was taken from each animal. Sampling all eight animals took ~ 45 min. Animals were then kept standing in the yard (no feed or water available) and a further saliva sample was taken from each animal at 12 noon. The cows were then grazed *ad libitum* on pasture from 1–2 pm, then food was withdrawn for a further 2 h, after which a third saliva sample was taken from each animal (4 pm). After this the animals were allowed access to short pasture

overnight. This routine was maintained for three successive days (days 1, 2, 3). The complete sampling procedure was repeated 7 days after the first sampling (days 8, 9, 10). Each saliva sample was analysed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and the amount of bSP30 in saliva was measured by scanning densitometry of the Coomassie brilliant blue-stained gels.

Saliva samples from the main herd

Based on our combined findings in the herd and time-of-day study above, saliva sampling on all animals in the main herd was carried out as described for the 9 am samples. HS and LS animals which grazed together were sampled together (one sample per animal). The herd consisted of different management groups (cows, yearlings, heifers, bulls and calves), each of which were sampled on different days.

Statistical analysis

Variability in the measurement of bSP30 abundance was estimated by performing 14 analyses of two standard samples, as well as 29 duplicate analyses of individual samples. Results from this and an experiment to measure day-to-day variation among animals were analysed using restricted maximum likelihood (REML) procedures.

Analysis of variance procedures were used to compare bSP30 abundances from animals sampled across the two herds using the GENSTAT statistical package (GENSTAT 5, Release 2·2, Lawes Agricultural Trust, Rothamsted Experimental Station, UK). Correlations of adjusted bSP30 relative abundance and adjusted mean bloat scores were calculated. bSP30 was adjusted for contemporary group (year of bloat scoring and sex) and gel number as fixed effects. For mean bloat score, the fixed effect was contemporary group (year of bloat scoring and sex).

Amino acid sequencing and amino acid composition analysis

The procedure used to obtain tryptic peptides of bSP30 was based on that of Aebersold (1993). Salivary proteins were subjected to SDS–PAGE as described above, then electroblotted to nitrocellulose membrane (Schleicher & Schull, Dassel, Germany). The transfer buffer contained 10 mM 3-[cyclohexylamino]-1-propanesulphonic acid (pH 11), and 10% (v/v) methanol. The blot was stained in a solution containing 0·1% (w/v) Ponceau S and 1% (v/v) acetic acid, then destained in water. The bSP30 band from 10 lanes was cut out, the pieces pooled and then incubated in 0·5% (w/v) polyvinylpyrrolidone MW 40 000 (PVP 40) (Sigma Chemical Co, St Louis, MO, USA) at 37°C for 30 min to prevent non-specific binding. The membrane pieces were washed five times with water and then cut into ~ 2 x 2 mm squares and incubated in 50 µl of a solution containing 50 mM Tris.HCl, pH 8·0, and 5% (v/v) acetonitrile. To this was added 10 µl (1 µg) L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (sequencing grade; Promega, Madison, WI) and the solution was incubated overnight at 37°C. The solution was then transferred to a fresh tube, leaving the membrane pieces behind. This solution was centrifuged to remove any particulates, acidified by adding 10 µl 10% (v/v) trifluoroacetic acid, and subjected to reverse-phase HPLC using a 5 mm diameter x 250 mm C-18 column (Zorbax; Rockland Technologies, Newport, DE). Individual peptide peaks were collected and three were subjected to amino acid sequencing using a gas-phase sequenator (model 470 A; Applied Biosystems, Foster City, CA).

Amino acid composition analysis was performed on a bSP30 band, which had been electroblotted onto polyvinylidene fluoride membrane (Immobilon P; Millipore, Bedford, MA), then stained with a solution containing 0·025% (w/v) Coomassie brilliant blue, 40% (v/v) methanol and 5% (v/v) acetic acid followed by destaining in 50% (v/v) methanol. A piece of membrane containing the bSP30 band was cut out, destained completely with methanol, dried and submitted to the Protein Microchemistry Facility, Department of Biochemistry, University of Otago, for amino acid composition analysis. The method used was acid hydrolysis followed by phenylthiohydantoin derivatization as described previously (Hubbard 1995). Detection was by UV absorbance. Cysteine and tryptophane were not quantified using this procedure.

Results

Analysis of salivary proteins from low and high susceptibility cows

At least seven major proteins were detected by electrophoresis of bovine saliva (Fig. 1).

Most of these proteins were present in similar levels in the saliva of animals from both LS and HS herds. However, several proteins appeared to vary in abundance between individuals. In particular, one protein appeared to differ consistently between the two herds (indicated with an

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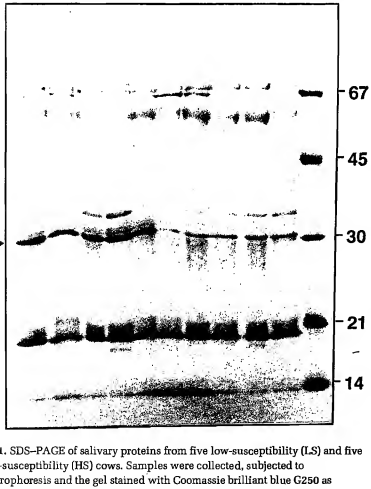
arrow in Fig. 1) and this protein is hereafter referred to as bovine s. .ry protein 30 kDa (bSP30).

Variability of bSP30 levels in bovine saliva with time

Saliva was sampled from eight cows subjected to the regimen described in the *Materials and methods*. Two of the HS and one of the LS cows were the same animals analysed in Fig. 1. Saliva samples were assayed for total protein as well as for relative abundance of bSP30.

Total salivary protein concentration did not vary significantly with time within an animal or between herds. Adjusted means for each time were: 9 am, 0·431 mg/ml; noon, 0·452 mg/ml; 4 pm, 0·444 mg/ml (standard error (SE) = 0·021 mg/ml) and for herd: HS, 0·464 mg/ml; LS, 0·421 mg/ml (SE = 0·096 mg/ml).

A standard deviation (SD) of 0·030 was obtained for measurement of bSP30 relative abundance using replicate analyses. This value includes both varia .y within the same gel (SD = 0·011) and variability between different gels (SD = 0·028). Mean values for the relative abundance of bSP30 are shown in Fig. 2a by herd and time. Averaging over the herds, the relative abundance was higher at 9 am than at 12 noon, by 0·022, (SE of difference = 0·006) and higher at 9 am than at 4 pm, by 0·048, (SE of difference = 0·006). The standard deviation of day-to-day variation over all eight cows was 0·024 (95% confidence interval (CI) 0·019–0·028). The SD within the HS cows was 0·018 (95% CI 0·013–0·022) and within the LS cows was 0·028 (95% CI 0·021–0·035). These values are significantly higher than the SD within a gel in replicate samples (0·011); therefore significant variation in bSP30 relative abundance from day to day within an animal was observed. This is illustrated for saliva collected at 4 pm from one animal from the LS and one animal from the HS herd in Fig. 2b. However, this variation was not as great as the difference between the herds.



1. SDS–PAGE of salivary proteins from five low-susceptibility (LS) and five h-susceptibility (HS) cows. Samples were collected, subjected to trophoresis and the gel stained with Coomassie brilliant blue G250 as cribed in the *Materials and methods*. bSP30 is indicated by the arrow.

Correlation of bSP30 levels in saliva with susceptibility to bloat

The relative abundance of bSP30 was plotted against the adjusted mean bloat score of each animal (see Fig. 3). The results indicate that LS animals tend to have a higher bSP30 relative abundance. The mean relative abundance of bSP30 was 0·255 for animals from the LS herd and 0·154 for animals from the HS herd, a 66% increase in the LS herd. The herd difference was 0·101 ± 0·022 ($P < 0·001$). Regression equations for the HS herd were $y = -0·82x + 1·40$ and for the LS herd $y = -2·39x + 0·82$, where y was average adjusted bloat score and x was adjusted relative abundance of bSP30. The overall regression line was $y = -3·37x + 1·50$. The correlation with bloat score was −0·40 ± 0·12 when herd of origin was not fitted. For each separate herd the correlations were −0·11 ± 0·15 (HS) and −0·34 ± 0·19 (LS) (Fig. 3). Therefore, the overall correlation with bloat score was significant but was not significant for animals within the HS herd and only marginally significant within the LS herd.

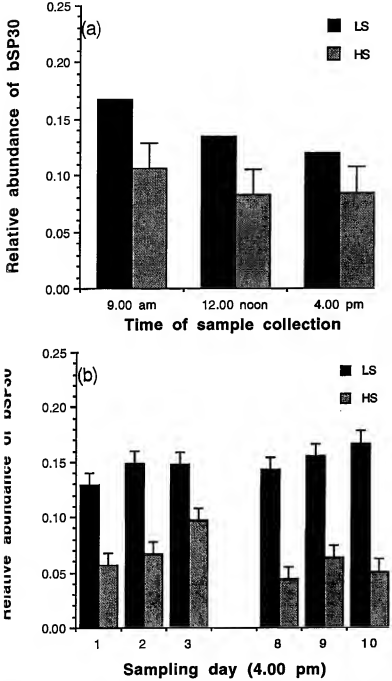
The relative abundance of bSP30 was tested for its suitability as a predictor of susceptibility to bloat in these animals. The optimal threshold value of bSP30 relative abundance for assigning animals to the LS and HS herds was between 0·19 and 0·24, where 70–85% of HS and 57–75% of LS animals were classified correctly.

Characterization of bSP

Experiments were conducted to further characterize bSP30. Salivary proteins from an HS and an LS animal were subjected to two-dimensional electrophoresis on ge-format gels. The gels revealed a limited number of highly abundant proteins (Fig. 4). One of these (arrowed) is a 30 kDa protein that is more abundant in saliva from the LS animal. This protein has an apparent pI of 5·3 on the gels.

In an effort to identify bSP30, proteolytic fragments of the protein, obtained after electroblotting from a one-dimensional SDS gel, were subjected to amino acid sequencing. The sequence of three peptides was obtained (VVVGEXPNNPESIS, IRELLESLDTETIK and LXGXPQVTTQQEXG), which together are likely to comprise about 20% of the amino acid sequence of a 30 kDa protein. An homology search using the BLAST program (NCBI, Bethseda, MD, USA) revealed no significant homologies to any protein sequences in the GENPEPT or SWISSPROT databases.

The bSP30 protein band was also subjected to amino acid composition analysis after blotting to polyvinylidene fluoride membrane. The relative amounts of individual amino acids comprising bSP30 are shown in Table 1. The relatively low proportion of proline indicates that bSP30 is not a member of the acidic proline-rich class of salivary proteins, which typically comprise more than 30% proline residues (Wallach *et al.* 1975).



g. 2. Variation in bSP30 relative abundance in saliva with time. (a) Effect of time 'day. Mean bSP30 abundance of four animals from the LS and four from the HS rd over 6 days is presented. Data were adjusted for effects of analysis on fferent gels using standard samples run on each gel. Error bars represent the andard errors for the differences between LS and HS (0·023) at each time of day.) Effect of day. The data are for one HS and one LS animal. Saliva from days 1, 2 id 3 were analysed on a single gel, as was saliva from days 8, 9 and 10. Error bars present the standard deviation of variation in bSP30 abundance in replicate alyses within a gel (0·011). Relative abundance of bSP30 was measured and iculated as described in Materials and methods.

Discussion

Our investigations of bovine salivary proteins have revealed a protein, bSP30, whose mean relative abundance was 66% higher in the LS herd compared with the HS herd. To our knowledge this protein has not been linked previously to bloat susceptibility. As bSP30 is an abundant protein, it is likely that it would have been detected in previous studies using native gel electrophoresis (e.g. McLaren *et al.* 1987). However, any variation in abundance may have been masked in earlier studies by comigration of other proteins, by the limited numbers of animals analysed, or by insufficient variation in the bloat susceptibility phenotype. The abundance of bSP30 may be under physiological control as it can vary significantly in an individual animal with time of day and from one day to the next. We have not investigated the effect of seasonality, lactation status, type of feed, the presence of clinical bloat or other environmental variables on bSP30 abundance. A high abundance of bSP30 is associated with low susceptibility to bloat, at least in the Ruakura bloat herds. However, the possibility that this has arisen through genetic drift between the LS and HS herds precludes any conclusion being drawn on whether the correlation holds

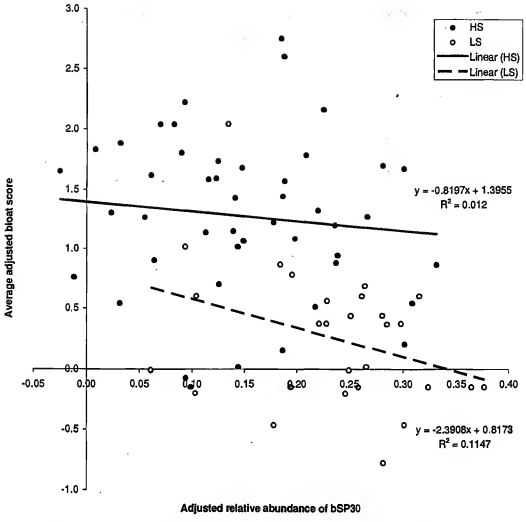
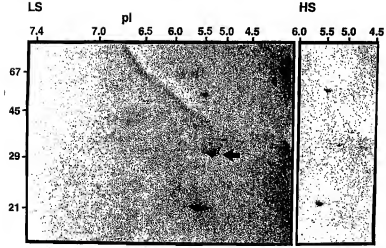


Fig. 3. Relationship of bloat susceptibility with bSP30 relative abundance. Using adjusted average bloat score without adjustment for herd, the correlation coefficient is –0·40 ± 0·12. The lines represent the regression lines for the data within each herd (solid line = HS herd, broken line = LS herd). Relative abundance of bSP30 was measured and calculated as described in the *Materials and methods*.



4. Two-dimensional electrophoretic separation of salivary proteins from an
und an HS cow. For the HS saliva gel, only the section of the gel containing
30 is shown. The putative bSP30 spot is arrowed.

true amongst the dairy cattle population in general. A conclusion on this must await experimental verification. Nevertheless, the herd difference in bSP30 abundance shows that it may be influenced by genetic factors.

Our initial characterization of the bSP30 protein shows that it is a relatively abundant protein in both parotid and mandibular salivary gland tissue (results not shown). Presumably it has a function in the salivary gland, in saliva or in its ultimate destination, the rumen. The amino acid sequence of three tryptic fragments of bSP30 revealed no homology with known proteins. bSP30 is an acidic protein (pI = 5·3 on two-dimensional electrophoresis gels). However, it does not appear to be an acidic proline-rich protein as it contains only 7% proline by amino acid composition analysis. The variability in abundance suggests a controlled physio-

Table 1. Amino acid compe[illegible]on of bSP30

Amino acid		% abundance
Asx	(B)	5·0
Glx	(Z)	5·4
Ser	(S)	5·6
Gly	(G)	5·5
His	(H)	4·7
Arg	(R)	6·2
Thr	(T)	6·2
Ala	(A)	6·0
Pro	(P)	7·1
Tyr	(Y)	6·6
Val	(V)	7·1
Met	(M)	5·8
Ile	(I)	7·3
Leu	(L)	6·9
Phe	(F)	6·2
Lys	(K)	8·4

Cys and Trp were not measured.
Glx = Glu + Gln and Asx = Asp + Asn

logical response.

One obvious application for a protein or DNA marker for susceptibility to bloat would be to screen dairy cattle to eliminate highly susceptible animals. However, even if the correlation we have observed were to hold true in animals outside the LS and HS herds, bSP30 has several undesirable characteristics in this regard. First, the loose correlation and degree of overlap in bSP30 levels in HS and LS animals limits the number of animals that could be definitively typed; second, the quantitative nature of the screen would require precise measurement of abundance; and third, the likelihood of a dependence of bSP30 abundance on environmental/physiological conditions and day-to-day variability would require careful control of these factors before taking saliva samples. If, however, the gene coding for bSP30, particularly the promoter region, were to contain differences in DNA sequence that are associated with the bloat susceptibility phenotype, then a genetic marker approach might be used. This would avoid most of the limitations described above. Despite the present limitations to its immediate application to the dairy industry, examining the association of bSP30 abundance with bloat susceptibility in the cattle population, in general, and further investigations into bSP30 function may lead to a better understanding of susceptibility to bloat in cattle.

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The BSP30 salivary proteins from cattle, LUNX/PLUNC and von Ebner's minor salivary gland protein are members of the PSP/LBP superfamily of proteins

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Abstract

Saliva influences rumen function in cattle, yet the biochemical role for most of the bovine salivary proteins (BSPs) has yet to be established. Two cDNAs (BSP30a and BSP30b) from bovine parotid salivary gland were cloned and sequenced, each coding for alternate forms of a prominent protein in bovine saliva. The BSP30 cDNAs share 96% sequence identity with each other at the DNA level and 83% at the amino acid level, and appear to arise from separate genes. The predicted BSP30a and BSP30b proteins share 26–36% amino acid identity with parotid secretory protein (PSP) from mouse, rat and human. BSP30 and PSP are in turn more distantly related to a wider group of proteins that includes lung-specific X protein, also known as palate, lung, and nasal epithelium clone (LUNX/PLUNC), von Ebner's minor salivary gland protein (VEMSGP), bactericidal permeability increasing protein (BPI), lipopolysaccharide binding protein (LBP), cholesteryl ester transfer protein (CETP), and the putative olfactory ligand-binding proteins RYA3 and RY2G5. Bovine cDNAs encoding homologs of LUNX/PLUNC and VEMSGP were isolated and sequenced. Northern blot analysis showed that LUNX/PLUNC, BSP30 and VEMSGP are expressed in bovine salivary tissue and airways, and that they have non-identical patterns of expression in these tissues. The expression of both BSP30a and BSP30b is restricted to salivary tissue, but within this tissue they have distinct patterns of expression. The proximity of the human genes coding for the PSP/LBP superfamily on HSA20q11.2, their similar amino acid sequence, and common exon segmentation strongly suggest that these genes evolved from a common ancestral gene. Furthermore, they imply that the BSP30a and BSP30b proteins may have a function in common with other members of this gene family.

Keywords: Parotid; Submandibular; BPI

1. Introduction

Saliva performs a range of digestion-related functions that include degradation of large food molecules, lubrication, protection of oral tissues, and maintenance of correct pH (reviewed in Ref. [1]). Components of saliva are secreted from a number of glands including the parotid gland, which in humans, secretes a protein-rich fluid of low viscosity and is thus referred to as a serous gland, and the submandibular and sublingual glands, which in humans secrete a carbohydrate-rich fluid of higher viscosity and are referred to as mucus glands [2]. Additional minor salivary glands include the von Ebner's gland. The proteins in saliva perform many of its functions [3,4]. The protein composition of saliva varies considerably among species (see Ref. [5] for a review), reflecting diverse diets and modes of digestion. The function of some of the salivary proteins is known; for instance the glycosidase, amylase; the protease, kallikrein; the histatin family of fungistatic proteins and the cystatin family of protease inhibitors [6–8]. The cDNAs coding for some salivary proteins have

Abbreviations: PCR, polymerase chain reaction; MOPS, 3-[*N*-morpholino]propanesulfonic acid; nt, nucleotide

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been cloned and sequenced [9–12]. These have revealed a wide diversity in primary sequence and extensive post-translational processing for salivary proteins [13]. But despite these advances, the primary structure and function of many of the salivary proteins still remains to be determined.

In cattle, saliva is a significant contributor to the rumen contents. Cattle have been estimated to produce up to 150 l of saliva per day [14,15]. Saliva contributes neutral buffering capacity, as well as enzymic and other activities that have not been well-characterised. We have previously investigated the role of saliva in influencing susceptibility to pasture bloat. An abundant protein was detected in bovine saliva, which we have termed bovine salivary protein 30 kDa (BSP30) [16]. Here we report the cloning and sequencing of two cDNAs, each containing a single open reading frame encoding different forms of BSP30, which we term BSP30a and BSP30b. These appear to be transcribed from two separate genes, and both have similarity with parotid secretory protein (PSP) from rat, mouse and human. The full-length BSP30a and BSP30b proteins were found to be structurally related to a group of 11 other proteins, including lung-specific X protein/palate, lung and nasal epithelium clone protein (LUNX/PLUNC) from human, rat and mouse, von Ebner's minor salivary gland protein (VEMSGP) from human and mouse, bactericidal permeability increasing protein (BPI), lipopolysaccharide binding protein (LBP), cholesteryl ester transfer protein (CETP), phospholipid transfer protein (PLTP), two putative rat olfactory tissue ligand-binding proteins, RYA3 and RY2G5, and three human hypothetical proteins. cDNAs encoding bovine homologs of LUNX/PLUNC and VEMSGP have been isolated and sequenced and used in Northern blot analyses along with the BSP30 genes. These results provide insight as to possible biological functions for the BSP30 proteins.

2. Materials and methods

2.1. cDNA library construction and cloning of full-length BSP30 cDNAs

PolyA$^+$ RNA was isolated from parotid tissue from a Holstein–Friesian cow (*Bos taurus*) using the guanidinium-acid phenol method [17] followed by affinity chromatography using oligo dT cellulose [18]. A 5 μg aliquot was used to construct a cDNA library in lambda phage using poly dT as the primer for first strand synthesis, and the uni-zap vector and cDNA synthesis kit and packaging mix obtained from Stratagene (La Jolla, CA). The manufacturer's instructions were followed throughout. A series of four degenerate oligonucleotides (15-mers) were synthesised based on a segment of one of the BSP30 sequences (PNNPE) each utilising either BGG or AAG as alternate codons for each of the two prolines. The four oligonucleotides were each end-labeled with ^{32}P using polynucleotide kinase following standard procedures [19], and used to probe Northern blots. One of the oligonucleotides (YTCBGGRTTRTTBGG) resulted in detection of a 950 nt band that was specific to parotid RNA (results not shown). This probe was then used to screen the amplified bovine parotid salivary gland cDNA library.

Full-length cDNAs for BSP30a and BSP30b were obtained by PCR from the library using two nested 3′ primers complementary to sequence within the BSP30 coding region (primer 1 ACCGTGAGTGAGATGCTTTC; nt 546–565, and primer 2 GAGATGCTTTCTGGGTTGTTG; nt 536–555 (see Fig. 1)) together with a primer complementary to sequence within the cloning vector (CGCTCTAGAACTAGTGGATC). DNA was extracted from approximately 5×10^5 plaque-forming units of the cDNA library by phenol-chloroform extraction [20] and used as template in a PCR reaction which contained 1 μM vector primer and primer 1, 10 mM Tris–HCl, pH 8.3, 2.5 mM $MgCl_2$, 50 mM KCl, 2.5 mM dNTP's, 60 μM tetramethylammonium chloride and 5 U Taq polymerase. The mixture was subjected to 30 cycles of amplification using annealing, extension and denaturing temperatures of 50, 72, and 94 °C, respectively, and a thirty-first cycle using 61 °C as the annealing temperature. A 0.5 μl portion of the 25 μl reaction was then used as template in a second round of 15 cycles containing the vector primer and primer 2 in the reaction mixture. The reaction mixture was subjected to electrophoresis, a 600 bp band was excised and cloned into a *t*-tailed Bluescript plasmid. The presence of BSP30a or BSP30b sequence was initially determined in 34 clones by restriction digests, of which 50% were found to contain BSP30a and the remainder BSP30b. Single-stranded DNA was then prepared from four BSP30a and four BSP30b clones and manual sequencing was performed with a Sequenase kit utilising an M13(-20) primer. Sequencing of the cDNA library clones was performed using an automated sequencer (ABI model 377) utilising primer sequences on the vectors.

cDNAs encoding the bovine homologs of LUNX/PLUNC and VEMSGP were identified following random sequencing of bovine cDNA libraries prepared from RNA extracted from the parotid salivary gland and tonsil, respectively. These clones were subjected to primer walking to obtain the full sequence of the cDNA. These sequences have been submitted to GenBank and have the accession numbers AF488706 and AF488705, respectively.

2.2. Northern analysis

RNA was isolated from bovine tissues using the guanidinium acid-phenol method [17] and 10 μg was subjected to electrophoresis on 1.2% (w/v) agarose gels containing 1 × MOPS buffer and 0.7% (v/v) formaldehyde [21]. After capillary transfer to nylon membrane, the RNA was UV-cross-linked and then stained with methylene blue [22]. Membranes were prehybridised in phosphate buffer [23]

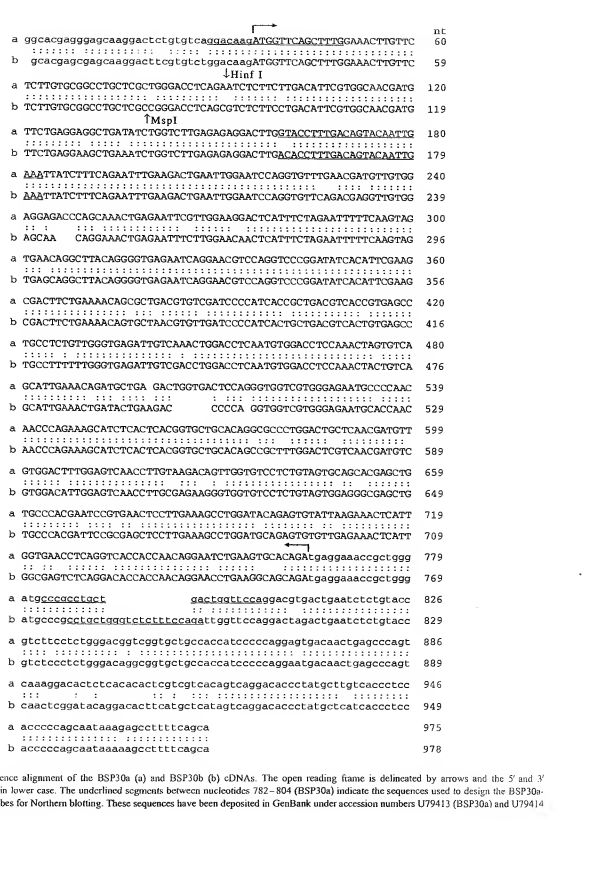

Fig. 1. Nucleotide sequence alignment of the BSP30a (a) and BSP30b (b) cDNAs. The open reading frame is delineated by arrows and the 5′ and 3′ untranslated regions are in lower case. The underlined segments between nucleotides 782–804 (BSP30a) indicate the sequences used to design the BSP30a- and BSP30b-specific probes for Northern blotting. These sequences have been deposited in GenBank under accession numbers U79413 (BSP30a) and U79414 (BSP30b)

then incubated overnight with ^{32}P-labeled probe. This was either at 65 °C with BSP30a, VEMSGP, or LUNX/PLUNC cDNA labeled using the random hexamer method, or at 35 °C with short oligonucleotides derived from either BSP30a or BSP30b (see Fig. 1) which were end-labeled with ^{32}P using polynucleotide kinase following standard procedures. Filters that were incubated with full-length cDNA probe were washed at 65 °C in 2× SSC. Filters that were incubated with the oligonucleotides were washed at 50 °C (BSP30a) or 40 °C (BSP30b) in 3× SSC before being exposed to X-ray film.

3. Results

3.1. Cloning and sequencing of cDNA coding for BSP30

We have previously reported the amino acid sequence of three peptides derived from the prominent bovine salivary protein, BSP30 after excision from an SDS polyacrylamide gel [16]. A bovine parotid salivary gland cDNA library was constructed and screened by filter hybridisation with the degenerate oligonucleotide YTCBGGRTTRTTBGG, which was derived from a segment of one of these sequences (PNNPE). Two positive hybridising clones contained inserts of 950 and 500 nt. These were sequenced and found to contain similar, but not identical sequences, designated BSP30a and BSP30b. Neither clone contained an initiation codon, so sequence 5′ to the end of the two BSP30 cDNAs was obtained by PCR amplification from the cDNA library as described in Materials and methods. The PCR products were then cloned and four clones were sequenced. Each sequence matched that predicted for BSP30a or BSP30b and extended the open reading frames sufficiently to include an initiation codon and 5′ untranslated region.

Sequence analysis revealed that the two clones are very similar but not identical at the nucleotide level (96.4% nucleotide sequence identity, Fig. 1). The predicted translation products, 243 and 240 amino acids in length for BSP30a and BSP30b, respectively, share 82.8% amino acid sequence identity, and the deduced translation products for both clones match with the amino acid sequence information obtained from three different BSP30 peptides [16]. There are five positions where extra nucleotides are present in one cDNA compared to the other, and for two of these (nt 514 of BSP30a and nt 496 of BSP30b; see Fig. 1), this results in a short frame shift over five (BSP30a) or three (BSP30b) amino acids. The most divergent part of the nucleotide sequence is located in the 3′ untranslated region and comprises a 13 nucleotide insertion in BSP30b relative to BSP30a. PCR across diagnostic MspI and HpaI sites in the BSP30a and b genes, respectively (primers indicated in Fig. 1) showed that BSP30a and b were both present in genomic DNA of all 18 Holstein–Friesian cattle tested (results not shown).

3.2. Sequence analysis of the PSP family members

A homology search using BLASTP against GenBank revealed four proteins that aligned to the BSP30a translation product with a significant score: human PSP (GenBank identifier AAL78113, which differs in a single ami-no acid residue from a human hypothetical protein XP_114180, 38% identity over the entire length); rat PSP (GenBank accession number B42337, 29% amino acid identity); mouse parotid salivary protein (GenBank accession number P07743, 27% amino acid identity) and rat submandibular gland protein (GenBank accession number A42337, 24% amino acid identity). The three rodent proteins are members of a group of salivary-specific proteins known as PSP [12,24]. Four amino acid sequences identical to XP_114180 were also obtained from the Derwent patent database (accession numbers W60682, W69221, B25765, B24069). Searches using PSI-BLAST [25] or SAM99 [26] identified three additional more distantly related proteins. These were the human and mouse homologs of the lung protein, LUNX [27] also referred to as PLUNC [28], a mouse VEMSGP and a hypothetical protein deduced from human genome sequence, protein XP_059283 (labeled X in Figs. 2 and 5), and its murine homolog BAB24760. A human homolog of VEMSGP was identified by BLAST analysis of GenBank using the mouse sequence. A rat homolog of LUNX/PLUNC has recently been described [29]. The sequence of bovine homologs for LUNX/PLUNC and VEMSGP were obtained by primer walking of two cDNAs derived from bovine parotid salivary and tonsil libraries.

A Clustal X multiple alignment was performed on these proteins (Fig. 2a), and their relationship with one another is shown as an unrooted tree (Fig. 2b). The tree shows BSP30a, BSP30b, human PSP, mouse PSP and rat PSP on a single branch, indicating that BSP30 is the bovine homolog of PSP. The branch lengths are consistent with a divergent family. All these proteins contain a highly conserved hydrophobic N-terminal region of 20 amino acids which corresponds to the signal sequence reported for mouse PSP. Assuming that this is removed in the mature BSP30 proteins, their predicted molecular weights and isoelectric points (BSP30a=24.4 kDa, p*I* 4.4 and BSP30b=24.7 kDa, p*I* 4.1) are similar but not identical to that of the BSP30 protein as analysed on one- and two-dimensional polyacrylamide gels (30 kDa, p*I* 5.0) [16]. There are a number of amino acids in common throughout the length of the proteins, including two cysteines separated by 42 amino acids, which have been previously identified as characteristic of the PSP family and are likely to be involved in disulfide binding [12] (see Fig. 2a). The predicted secondary structure of the proteins, obtained using the method of Rost and Sander [30], was very similar despite their low sequence similarity. Several regions of alpha helix at the N- and C-terminal ends (residues 1–90, and 180 to the end) surrounding a region

of beta sheet aligned well, while the predicted loop structures did not align well (result not shown). These features, along with their similar sequence length, clearly place BSP30a and b, PSP, LUNX/PLUNC, and the human hypothetical protein XP_059283 in the same group, despite the low overall sequence identity.

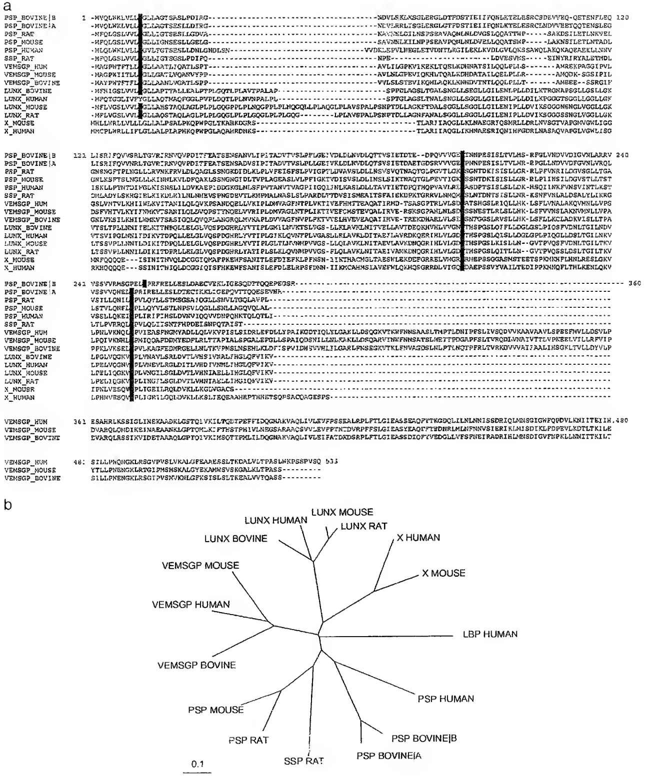

Table 1
Nomenclature for PSP family members

Protein name (reference)	Alternative names (reference)	GenBank accession number
PSP (10)	SPLUNC 2 (32) BSP30	human: XP_114180, AAL28113, AF432917 mouse: P07743 cattle: U79413, U79414 rat: B42337
LUNX/PLUNC	PLUNC (28) LUNX (27) SPLUNC 1 (32)	human: BAA93633, BC012549, AP172993, AF214562 mouse: AAB63256, NM_011126, AF356785 cattle: AF488706 rat: AAM73687
VEMSGP	LPLUNC 1 (32)	human: XP_047037, NP_149974, U46068, AF364078 mouse: AAA87581 cattle: AF488705
RY2G5 (40)	LPLUNC 4 (32)	human: CAC18887 mouse: BB559536 rat: CAA43067, X60660
RYA3 (40)	LPLUNC 3 (32)	human: CAC18886, NM_025227, AL121756 mouse: AV251825 rat: X60658
X	SPLUNC 3 (32)	human: XP_059283 mouse: BAB24760
Y		human: XP_066207
Z	LPLUNC 2 (32)	human: CAC18884

The human, mouse and bovine VEMSGP protein sequences are significantly longer than the PSP proteins, suggesting the presence of an extra domain. The mouse VEMSGP amino acid sequence was therefore used to perform an additional round of PSI-BLAST and SAM99 searches of GenBank for further related proteins. This revealed structural similarity to eight additional proteins (GenBank identifiers given for human homologs only): BPI (GenBank accession number XM_059336); LBP (NM_004139); PLTP (AB076694); CETP (NM_000078); two putative ligand binding proteins found in rat olfactory tissues, RYA3 and RY2G5 (S17448 and S17447); and two human hypothetical proteins XP_066207 and CAC18884 (referred to in Fig. 5 as the Y- and Z-genes, respectively). BPI, LBP, PLTP and CETP have been referred to as members of the LBP family [31]. A recent report [32] also refers to some of the human homologs of these genes as SPLUNC1 (LUNX/PLUNC), SPLUNC2 (PSP), SPLUNC3 (hypothetical protein XP_059283), LPLUNC1 (VEMSGP), LPLUNC2 (hypothetical protein CAC18884), LPLUNC3 (RYA3) and LPLUNC4 (RY2G5). Table 1 list each of the genes and their GenBank nucleotide or amino acid accession numbers. VEMSGP and the additional eight proteins each contain two protein domains (SMART BPI1 and BPI2) recognised by the Conserved Domain Database Search at NCBI [33]. The N-terminal of these domains (BPI1) was also detected in BSP30a and the XP_059283 human hypothetical protein. However, the statistics of these associations for VEMSGP, CAC18884, BSP30a and XP_059283 were weak (E>1e − 4) compared with the corresponding domains in the other proteins (E < 1e − 40). The PSP family members and representatives of the other families were then submitted to MEME analysis [34]. Three motifs detected by MEME were common to all sequences examined. One corresponds to the signal sequence and the other two with consensus sequences, KDETGRPHLVMGRCSHNPGSIHISLLHRR and VMNLLRRVLPHVVQNQLCPVIQYILYQMD, which contain the conserved cysteine pair

Fig. 2. (a) Clustal X alignment produced by the neighbor-joining method. The conserved cysteines are shaded. (b) Unrooted tree derived from the alignment. Human lipopolysaccharide binding protein (LBP_HUMAN, GenBank P18428) has been added as an outbranch. GenBank identifiers for the sequences. PSP_BOVINE|A (BSP30a), AAB38282; PSPBOVINE|B (BSP30b), AAB28283; PSP_HUMAN (parotid secretory protein), AAL28113, PSP_RAT, B42337; PSP_MOUSE, P07743; SSP_RAT (submandibular salivary protein), A42337; VEMSGP_BOVINE (von Ebner's minor salivary gland protein), AF488705; VEMSGP_HUMAN, NP_149974; VEMSGP_MOUSE, AAA87581; LUNX_BOVINE (LUNX/PLUNC) AF488706; LUNX_HUMAN, BAA93633; LUNX_MOUSE, AAB63256; LUNX_RAT, AAM73687; X_HUMAN (hypothetical protein XP_059283); X_MOUSE, BAB24760. The scale represents the distance corresponding to mean number of differences per site within an alignment. 0 = all identical, 1.0 = all different.

described above and are separated by approximately 10 residues, depending on the sequence examined. When these two domain sequences were used in a MAST search against the non-redundant database maintained at UCSD (mimc. sdsc.edu), a number of significant hits were obtained to members of the PSP, and LBP families, VEMSGP, RYA3, RY2G5 and the XP_059283, CAC18884 and XP_066207 hypothetical proteins with no evidence of false positives within the set returned (results not shown). Taken together, these results strongly suggest that the BSP30a and b, LUNX/PLUNC, and XP_059283 are structurally related to this wider group of proteins.

3.3. Expression of bovine PSP homologs

The expression of the two BSP30 genes, the bovine LUNX/PLUNC and bovine VEMSGP was examined in different tissues. RNA was extracted from a range of bovine tissues and analysed by Northern blotting using BSP30b cDNA, which hybridises to both forms of BSP30 mRNA, as a probe. A prominent band of approximately 950 nt was observed in parotid and submandibular salivary tissue. This size is similar to those predicted from the cDNA sequences of 975 nt for BSP30a and 978 nt for BSP30b, indicating that the sequences obtained are very close to full length. No

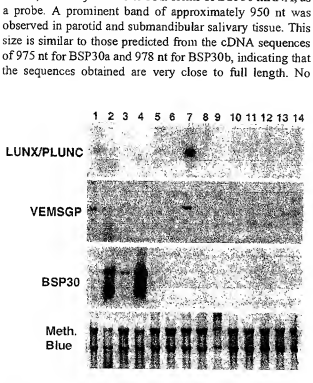

Fig. 3. Levels of BSP30, LUNX/PLUNC and VEMSGP mRNAs in a range of bovine tissues. RNA was isolated from the following bovine tissues; (1) nasopharyngeal, (2) parotid salivary, (3) sublingual salivary, (4) submandibular salivary, (5) lung, (6) tongue, (7) trachea, (8) adrenal, (9) brain, (10) heart, (11) liver, (12) kidney, (13) muscle, and (14) spleen. The RNA was subjected to triplicate Northern blot analysis (lanes 1 to 14 in the order of tissues listed above) with the membranes being stained for RNA with methylene blue (fourth panel) before being probed with full-length bovine LUNX/PLUNC (first panel), bovine VEMSGP (second panel) or BSP30b cDNA (third panel). After hybridisation and washing, the membranes were exposed to film for 16 h (LUNX), 2 days (VEMSGP) or 4 h (BSP30). The position of the bands on the blots were as expected for bovine LUNX (1049 nt), bovine VEMSGP (1624 nt) and BSP30b (950 nt).

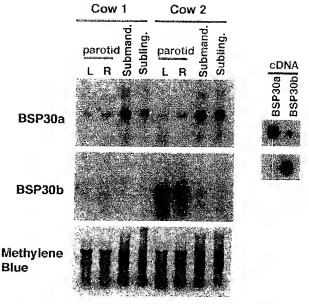

Fig. 4. Northern analysis of salivary gland RNA from two cattle using the probes specific for BSP30a or BSP30b. RNA was isolated from the left (L) and right (R) parotid, submandibular and sublingual salivary gland tissue. After transfer to membrane, the blot was stained for RNA with methylene blue (bottom panel). Duplicate membranes were probed for BSP30a (top panel) or BSP30b (middle panel) using two oligonucleotide probes BSP30a (tggaccagtcagcaggcggg) and BSP30b (tctggaaagagaccacgcagg) as indicated in Fig. 1. BSP30a and BSP30b cDNA was spotted onto membrane, and hybridised with the BSP30a (upper small panel) and BSP30b (lower small panel) probes, then washed as described in Materials and methods. Washed membranes were exposed to X-ray film for 3 days (BSP30a) or 4 days (BSP30b).

detectable expression was observed in nasopharyngeal, lung, tongue, trachea, adrenal, brain, heart, liver, muscle and spleen, and only a very low level of expression in the kidney (Fig. 3). A smeared signal was observed below the 950 nt band, most likely due to partial mRNA degradation. In contrast, expression of LUNX/PLUNC was restricted to the trachea and nasoepithelial tissue, while VEMSGP was expressed in trachea, nasoepithelial tissue, as well as in salivary tissue. BSP30a and BSP30b expression was further examined in bovine parotid, submandibular and sublingual salivary glands using BSP30a- and BSP30b-specific oligonucleotide probes derived from the region surrounding the 13 bp insertion/deletion (see Fig. 1). Using the BSP30a-specific probe, a 950 nt band was observed in all the salivary glands of both animals tested, while a 950 nt band was observed predominantly in the parotid glands of only one of the animals when the BSP30b-specific probe was used (Fig. 4). Parallel analysis of BSP30a and BSP30b cDNA indicated only minor cross-hybridisation using the BSP30a probe and essentially none using the BSP30b probe (Fig. 4, small panels). These results show that expression of the genes coding for these members of the PSP group of proteins is restricted to particular tissues, and that these are different for each member of the group that was tested.

4. Discussion

The cDNAs coding for two alternative forms of the major bovine salivary protein, BSP30 have been isolated and sequenced and the amino acid sequence of the proteins deduced. The number of differences between the BSP30a and BSP30b sequences and their presence in all 18 individual animals tested suggests they are unlikely to be alleles of a single gene, but rather are two separate genes that arose by duplication. The amino acid sequences of BSP30a and BSP30b share 20–30% identity with mouse and rat PSP, and rat submandibular gland protein. In addition, they contain two cysteines which are conserved among PSP, VEMSGP, LUNX/PLUNC and the hypothetical protein X (XP_059283) from several species. BSP30a and BSP30b are thus clearly related to these proteins, and BSP30 is most likely to be the bovine ortholog of rodent and human PSP. The two BSP30 genes, VEMSGP and LUNX/PLUNC each have a unique pattern of expression in different bovine tissues, consistent with reports of the expression of their homologs in other species [28,32,35]. The salivary gland-specific but non-identical expression of BSP30a and b suggest they may have distinct functions. BSP30b expression seems to be more restricted than BSP30a.

The NCBI (www.ncbi.nlm.gov) and UCSC (genome.ucsc.edu) Human Genome browsers were used to determine the genomic location of the human homologs of the 12 members of the PSP/LBP superfamily. All but one of these genes were found to reside on the q arm of human chromosome 20. PSP, LUNX/PLUNC, VEMSGP, RYA3, RY2G5, and the hypothetical proteins XP_059283, X_066207 and CAC18884 were contiguous over a region of 300 kbp (Fig. 5). The genes for LBP and BPI were found to be adjacent to each other, approximately 5 mbp distal to the PSP cluster. The PLTP gene was a further 7.5 mbp distal to

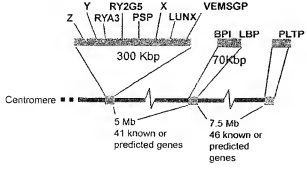

Fig. 5. Schematic diagram of the PSP/LBP superfamily gene cluster on HSA20q11.2. Gene X is hypothetical protein XP_059283, gene Y is hypothetical protein XP_066207 and gene Z is hypothetical protein CAC18884. The positions of the genes were deduced from the sequence of BAC clones RP11-49G10, RP4-726C3, RP4-733023, and RP5-1187J4, from a 5 mbp segment of working draft sequence (NT_028392), as well as using the NCBI genome browser, MapViewer (www.ncbi.nlm.gov).

the LBP, BPI genes. The CETP gene was the only one of the superfamily of proteins not present in the gene cluster; its gene resides on chromosome 16. These observations are consistent with earlier reports suggesting that BPI, LBP, PLTP and CETP (the LBP group of proteins) are structurally related [31], and that human homologs of the above genes, as well as PSP, LUNX/PLUNC, VEMSGP, RYA3, RY2G5, and the hypothetical proteins XP_059283 and CAC18884 are related to each other and present as a cluster on HSA20 [32]. Our results add the two bovine PSP homologs (BSP30a and b) and the human hypothetical protein XP_066207 to this superfamily, thus providing insight into their possible function. The UCSC Genome browser was also used to show that the same order of these genes occurs on mouse chromosome 2. Only a single PSP gene was detected in both human and mouse suggesting that duplication of the PSP gene appears to have occurred after evolutionary divergence of cattle from rodents and human. The genomic and cDNA sequences of the human homologs of the genes were used to predict their exon segmentation using the Spidey program (www.ncbi.nlm.gov/IEB/Research/Ostell/Spidey). As recently reported [32], all the PSP family members (PSP, LUNX/PLUNC, and hypothetical protein XP_059283) contained internal exons that were of similar size between the proteins, whereas the intron sizes and the overall gene sizes were highly variable (data not shown).

The function of some members of the PSP/LBP superfamily is known. BPI, LBP, PLTP and CETP have been shown to bind lipopolysaccharides, phospholipids and cholesteryl esters, respectively. BPI and LBP are involved in the response to bacterial infection [36], while PLTP and CETP are involved in lipid transport in plasma [37–39]. The rat olfactory proteins RYA3 and RY2G5 are thought to be involved in oderant binding [40]. It has been suggested that LUNX/PLUNC is either a secreted morphogen or a component of mucus [28], and it has also been identified as a marker of airways irritation [41] and non-small cell lung micrometastasis [21]. To our knowledge, no published information on the function of VEMSGP is available. BPI is a membrane-associated protein present in the secretory granules of neutrophils, and has cytotoxic activity against a range of gram-negative bacteria [42]. Cytotoxicity is mediated through the N-terminal portion of the protein [43]. Crystal structure analysis has shown that this domain forms a beta barrel structure that binds to and neutralises lipopolysaccharides on the outer membrane of gram-negative bacteria, and that a non-polar phosphatidylcholine binding site is present on the dorsal surface of the domain [44]. This N-terminal region of BPI appears to share some degree of structural relatedness with BSP30a and b. Furthermore, mouse PSP has been shown to bind to bacteria and bacterial membrane proteins in vitro [45]. It is therefore possible to speculate that BSP30 may also have a bactericidal function, either in the oral cavity or in the rumen.

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